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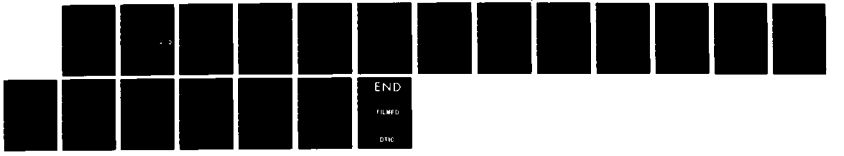

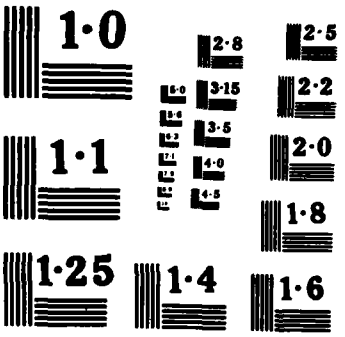

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20. The control of T and B lymphocyte responses is a major goal of immunobiology. In this study we have examined the control of B cell responses in vitro by several lymphokines including IL-1, IL-2 and gamma interferon. This analysis provides information on the potential to manipulate B cells in culture for their production of desired antibodies, as may be needed prior to hybridoma production and also provides information on the mechanism of regulation of lymphocyte responses.

In the present study we have shown that IL-1 and more importantly gamma

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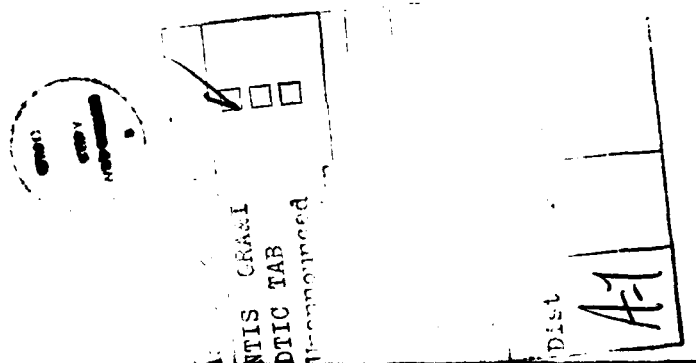
interferon are involved in the regulation of B cell responses. The role of gamma interferon in T cell-replacing factor (TRF) activity for antigen-specific plaque-forming cell (PFC) responses in vitro was studied using antibodies to murine gamma interferon. TRF activity was present in supernatants (Sn) of Con A- or mixed leukocyte reaction-stimulated murine spleen cells as well as in an IL-2-rich fraction of phytohemagglutinin-stimulated murine human peripheral blood lymphocyte Sn and in the Sn of the Gibbon T lymphoma MLA-144. The human TRF was highly active with cells from nu/nu mice and normal mice but not with cells from animals with the xid immunologic defect, similar to the activity of murine TRF. Antibodies to gamma interferon consisted of hyper-immune rabbit antisera, gamma interferon affinity-purified rabbit immunoglobulin and an interspecies hybridoma specific for murine gamma interferon. The results show that the activities of all preparations of TRF are markedly diminished or abrogated by antibody to murine gamma interferon but not by antibodies to human gamma interferon, nor by normal rabbit sera or purified rabbit Ig. The degree of inhibition was dose dependent and was quantitatively reversed by the addition to the cultures of recombinant-derived murine gamma interferon but not human gamma interferon. This reversal was fully antigen specific and thus not attributable to polyclonal B cell activation by gamma interferon, which is inactive alone in the TRF assay. Kinetic analysis shows that gamma interferon must act by 24-48 h to produce PFC responses at 4 d. Together, the data demonstrate that gamma interferon is a necessary mediator for TRF effects and that gamma interferon is induced by TRF from T-depleted murine spleen cells in sufficient quantity to support large antibody responses. The source of this gamma interferon may be the potent natural killer cells that are induced in cultures stimulated with TRF.

INTRODUCTION

Antibody responses can be induced in vitro from B cell populations with a variety of stimuli, provided that T cells or various cytokines are present in the cultures. In most studies, initial signals for B cell activation are generated via immunoglobulin (Ig)[3] receptors and are mediated by antigens or by anti-Ig antibodies (1-4). Subsequent B cell proliferation and differentiation to antibody secreting cells is greatly augmented by the presence of soluble mediators generated in cultures containing activated T cells. Early studies described a T cell replacing factor (TRF), present in supernatants (Sn) of murine mixed leukocyte reactions (MLR), which supported antibody responses of spleen cells from nu/nu mice to sheep red blood cells (SRBC) (5). Subsequent work revealed a similar activity in supernatants of concanavalin A (Con A)-stimulated spleen cells which was not active on cells from mice with the xid defect (6). Similar TRF activity for murine B cells has been shown with human MLR supernatants (7), suggesting that the mediators can also function across species barriers.

Early efforts to characterize the molecular components of TRF met with little success, possibly since several mediators cooperated in the biological assays. Recent work has attempted to analyze the specific cytokines using supernatants of monoclonal or long-term T cell lines and lymphokine fractions of known biological activity. Results from these studies show that at least two different lymphokine preparations act synergistically to produce a TRF effect on B cells in plaque forming cell (PFC) responses driven either by antigen or anti-Ig (8-10). One of the complementing components is usually obtained from T cell hybridomas and contains interleukin-2 (IL-2). The other is obtained in supernatants of a long-term alloreactive T cell line or some T-T hybridomas and contains several biological activities including interferon-γ (IFN-γ), and is termed T cell replacing factor (TRF) or interleukin-X (IL-X) (11) and is active in responses to hapten-carrier antigens (12) as well as thymus-independent antigens (13). It is possible, however, that both supernatants contain other mediators which may contribute to the antibody responses and some studies indicate that interleukin-1 (IL-1) (11) and perhaps other factors (10) may be required to obtain maximum levels of TRF activity. However, the precise role for any of these factors in the antibody response is not known and the target cells for their actions remain to be identified.

Results from some recent studies have suggested that IFN-γ may be a component of TRF. Indirect evidence was obtained from the acid-labile character of TRF present in antigen or Con A-stimulated T cell supernatants (1,12) and also from a correlation of IFN-γ levels and TRF activity of supernatants of T cell hybridomas (14). This view is supported by recent data showing that recombinant-derived murine IFN-γ (r-mo-IFN-γ) can act as the second component of a complementing TRF system to support PFC responses to antigen (15). However the specificity of the IFN-γ effect is unclear since it may perhaps also act as a polyclonal activator for B cell PFC responses (16). Together the data suggest that purified IFN- may modulate B cells in their maturation to antibody production, however it is also not clear whether this is accomplished through the pathway normally regulated by TRF or a different one. Similarly, it is possible that Con A or MLR supernatants contain other TRF mediators, distinct from IFN-γ, which could independently support PFC responses.



In the present study we have tested whether IFN-γ is essential in the TRF activity of four independent TRF preparations, including two which are derived from primate cells. This was accomplished by adding to the cultures antibody to IFN-γ which has been shown to neutralize the biological activity of IFN-γ in antiviral assays and macrophage activation assays (17,18) and similar inhibitory effects have also been obtained in Ia antigen-induction systems (19). The results clearly show that IFN-γ is a required mediator in the action of several forms of TRF, that it only acts relatively early in the response and that, in cases of TRF which lack this factor, the IFN-γ is produced by the responding cells, despite extensive T cell depletion. The source of the IFN-γ maybe the natural killer (NK) cells which are produced in these cultures.

TRF activity of an IL-2-rich lymphokine fraction. In preliminary experiments we found that a mitogen-free fraction of the supernatant of activated human peripheral blood mononuclear cells (PBMC), termed TRF(E-N), acted as a potent TRF for murine spleen cells. The cells were prepared from mice which had received ATS in vivo, and were treated twice with monoclonal anti-Thy-1.2 and C' and depleted of plastic- and Sephadex G-10-adherent cells. As shown in Figure 1, TRF(E-N) reconstituted the PFC response to SRBC of splenic B cells from (DBA/2xCBA/N)F_1 male mice but not that of the reciprocal hybrid. TRF(E-N) showed considerable helper activity at concentrations ranging from 1-5% in the culture and was only moderately dependent on the addition of exogenous IL-1, perhaps owing to the cell density of the cultures. In almost all experiments the above methods produced high PFC responses to SRBC, generally from 1,000 to 1,500 PFC per culture. Similar results were obtained following more stringent T-cell depletions, in addition to the ones described. These included in vivo and in vitro treatment with anti-asialo GM_1 serum, and in vitro treatment with monoclonal anti-Lyt-1, Lyt-2, Qa-4, and Qa-5 antibodies in the presence of appropriate enhancing sera and C', followed by B cell enrichment by positive selection for Ig-bearing cells (Table A). As shown below, PFC responses which were supported by TRF(E-N) were fully antigen-specific and were also generated with spleen cells from nu/nu mice (not shown). Thus human TRF(E-N) exhibits properties concordant with those of the previously characterized murine TRF preparations.

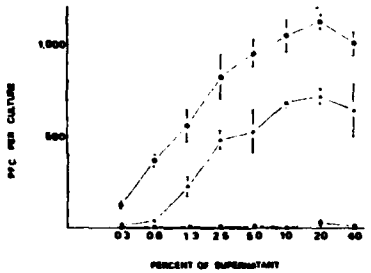


FIGURE 1 Response of normal and immunodeficient C3BA/N hybrid mice to human-derived TRF(E-N) and the effect of IL-1. T-depleted spleen cells were prepared from (DBA/2 × C.BA/N)F$_1$ (Δ) or (C.BA/N × DBA/2)F$_1$ (■) male mice and cultured for 4 d in the presence of SRBC, 2×10^6 per well, and TRF(E-N). IL-1 at 1.0 U per culture was added to the (DBA/2 × C.BA/N)F$_1$, (●) and (C.BA/N × DBA/2)F$_1$ culture. No PFC were obtained with the latter group in the absence of IL-1. Direct PFC to SRBC were determined at 4 d and expressed as the mean of triplicate cultures ± SEM. Control values, mean (SEM): spleen cells alone, 5 (3); plus SRBC, 0; plus TRF(E-N), 7 (7).

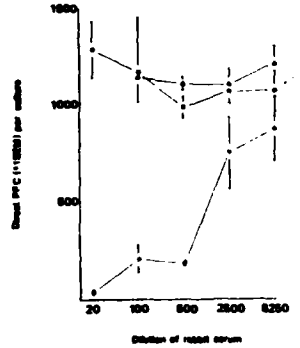


FIGURE 2 Inhibition of TRF-induced PFC by antiserum to Mu IFN-γ. T-depleted spleen cells from (DBA/2 × C.BA/N)F$_1$ mice were cultured for 4 d in the presence 20% TRF(E-N) and SRBC. Rabbit antisera were added at the start of the cultures at the indicated final dilutions. The sera are from (■) rabbit M13 before immunization, (●) rabbit M13 hyperimmunized with Mu rIFN-γ, and (Δ) rabbit 12R1 hyperimmunized with Hu rIFN-γ. A 1:20 dilution of M13 antiserum is equivalent to 625 IFN-γ viral neutralizing units per culture. Control values: spleen cells alone, 0; plus SRBC, 15 (6); plus TRF(E-N), 16 (4); plus TRF(E-N) and SRBC, 1,569 (211).

TABLE A

Phenotypic analysis of responding spleen cell preparations at the start and end of TRF assays using three methods for T cell depletion.

Time of analysis	Culture condition	Percent antigen-positive cells using method 1 Thy-1	1 Mlg	2 Thy-1	2 Mlg	3 Thy-1	3 Mlg	4 Thy-1	4 Mlg
Start of culture		25.7	62.5	0.6	93.9	0.4	91.4	0.3	89.3
End of culture	TRF(E-N)	30.0	74.8	7.8	95.5	9.1	98.6	4.2	99.0
	TRF(E-N)+ SRBC	28.6	78.4	7.5	97.9	11.2	99.2	5.5	99.5

PFC RESPONSES	PFC per culture(SEM) 1	2	3	4
TRF(E-N)	23(12)	88(40)	163(52)	87(38)
SRBC + TRF(E-N)	860(51)	1808(85)	990(56)	813(101)

Four cell preparations were examined at the begining of culture and after the 4 day culture period. The cells were cultured at 10^6 cells per well with the indicated additions. Analysis was carried out by staining the cells with directly conjugated, anti-Thy 1.2 (B-D) or anti-Mlg (Cappel). The cells were then analysed using an Ortho Cytofluorograph System 50-H, and an Ortho Diagnostics 2150 computer, the results are expressed as percent positive cells (10,000 cells examined). PFC were determined on day 4 and results are expressed as mean PFC(SEM) per culture.

Method of cell preparation:

1. Spleen cells no treatment.
2. ATS in vivo, anti-Thy 1.2, anti-Lyt-1 and anti-Lyt-2 in vitro and complement with enhancing serum added as a second step in vitro (the treatment was done twice).
3. ATS and anti-asialo GM_1 in vivo, anti-Thy 1.2, anti-Lyt-1, anti-Lyt-2, anti-Qa-4, anti-Qa-5 in vitro and complement with enhancing serum added as a second step in vitro (the treatment was done twice) the second first antibody treatment also had the addition of anti-asialo GM_1.
4. ATS and anti-asialo GM_1 in vivo, anti-Thy 1.2, anti-Lyt-1, anti-Lyt-2, anti-Qa-4, anti-Qa-5 in vitro and complement with enhancing serum added as a second step in vitro (the treatment was done twice) the second first antibody treatment also had the addition of anti-asialo GM_1. The Ig^+ cells were then purified on plastic dishes coated with affinity-purified goat anti-murine $F(ab')_2$, the adherent cells were recovered with 0.025% trypsin.

Antibodies used:

1. Anti-Lyt-1 and Lyt-2 (53-7.3 and 53-6.7) were used at a final concentration of 1:1000.
2. The enhancing serum was a rabbit anti-rat IgG (Cappel) and was used at its calibrated optimum of 1:50.
3. Anti-asialo GM_1 (Wako Chemicals) was given at 0.4ml of a 1:10 dilution i.v., and in vitro it was used at a final concentration of 1:20.
4. Anti-Qa-4 and Qa-5 (28-4.1, R. Cook, and B16-167, U.Hammerling and G. Koo) was used at a final concentration of 1:100.

TABLE I

Antibody to IFN-γ Inhibits the TRF Activity of Murine MLR Sn and TRF(E-N)

Antigen	Purified antibody to Mu IFN-γ (ng/well)	PFC per culture (SEM)	
		TRF (MLR)	TRF(E-N)
SRBC	0	1,087 (92)	1,038 (42)
SRBC	250	57 (17)	90 (12)
SRBC	83	425 (84)	667 (58)
SRBC	28	615 (52)	895 (152)
SRBC	9	680 (81)	937 (109)
	Normal Ig (ng/well)		
Controls			
SRBC	1,000	1,060 (49)	907 (66)
—	—	10 (6)	55 (10)

Murine T-depleted spleen cells were prepared as described in Materials and Methods except plastic adherence and Sephadex passage was omitted. 10^6 cells were cultured for 4 d in the presence of 25% murine or 20% human TRF(E-N) Sn. Immunospecifically purified antibody to Mu rIFN-γ was added at the start of the cultures. MLR Sn was the supernatant of a 24 h murine MLR. Antibody was present from the start of the cultures and had 10 U of antiviral neutralizing activity per 100 ng.

TABLE II

Antiserum or Specific Antibody to IFN-γ Inhibits the Action of Human TRF(E-N) or Murine (Con A) TRF on B Cell Responses to SRBC and Reversal of the Effect with Mu rIFN-γ

Experiment 1 TRF	Antigen	Antiserum to murine IFN-γ (units)	Addition of Mu rIFN-γ (2,500 U)	PFC to SRBC per culture (SEM) of 10^6 B cells
Con A/Sn	SRBC	0	–	1,020 (70)
Con A/Sn	SRBC	50	–	157 (55)
Con A/Sn	SRBC	6.3	–	205 (77)
Con A/Sn	SRBC	3.1	–	315 (72)
Con A/Sn	SRBC	1.6	–	290 (100)
Con A/Sn	SRBC	0.8	–	583 (198)
Con A/Sn	SRBC	0	–	920 (52)
Con A/Sn	SRBC	25	–	210 (55)
Con A/Sn	SRBC	25	+	750 (107)
Con A/Sn	No SRBC	0	–	25 (9)
TRF(E-N)	SRBC	0	–	967 (111)
TRF(E-N)	SRBC	25	–	425 (50)
TRF(E-N)	SRBC	25	+	935 (72)
TRF(E-N)	No SRBC	0	–	12 (6)
None	SRBC	0	–	7 (2)

Experiment 2 TRF	Antigen	Purified antibody to Mu IFN-γ (ng)	Addition of Mu rIFN-γ (2,500 U)	PFC to SRBC per culture (SEM) of 10^6 B cells
TRF(E-N)	SRBC	0	–	1,290 (64)
TRF(E-N)	SRBC	1,000	–	87 (33)
TRF(E-N)	SRBC	500	–	267 (101)
TRF(E-N)	SRBC	250	–	557 (142)
TRF(E-N)	SRBC	125	–	883 (151)
TRF(E-N)	SRBC	63	–	1,013 (219)
TRF(E-N)	SRBC	500	+	1,243 (87)
		Normal rabbit Ig (ng)		
TRF(E-N)	SRBC	1,000	–	1,350 (55)
None	SRBC	0	–	0
None	None	0	–	0
TRF(E-N)	None	0	–	23 (12)

T-depleted spleen cells were cultured at 10^6 cells per microculture for 4 d in the presence of 25% murine Con A Sn or 20% human TRF(E-N) Sn. Mu rIFN-γ (Exps. 1 and 2), rabbit antiserum (Exp. 1), or immunospecifically purified Ig (Exp. 2) were added at the indicated concentration per well, at the initiation of the cultures. 1,000 ng of antibody had 100 antiviral neutralizing units of activity.

Rabbit antiserum to mo-IFN-γ inhibits TRF(E-N) action. The requirement for IFN-γ as a mediator in these spleen cell responses was tested by adding to the cultures a hyperimmune rabbit antiserum to highly purified recombinant-derived murine IFN-γ. As shown in Figure 2, the addition of 5% rabbit antiserum to mo-IFN-γ totally inhibited the production of PFC. A substantial inhibition was still seen at 0.2% (1/500). The IFN-γ-neutralizing activity at this latter dilution was 25 units per microculture (0.20 ml), which was selected for routine use in most subsequent experiments for reasons of economy. Control rabbit serum, obtained from the same animal prior to immunization, was inactive and serum from a rabbit immunized to recombinant-derived human IFN-γ ($8x10^4$U per ml) also had no effect.

Inhibition of murine TRF(E-N) by specific Ig or monoclonal antibody and reversal with r-mo-IFN-γ. Confirmation that antibody to IFN-γ was the active component in the antiserum to IFN-γ was obtained with an affinity-purified antibody eluted from a column of unsolubilized recombinant-derived murine IFN-γ. As shown in Table I the addition of purified anti-IFN-γ antibody inhibited responses of spleen B cells whereas normal rabbit Ig did not. In this experiment TRF from both a murine MLR as well as the human-derived TRF(E-N) were inhibited by antibody to mo-IFN-γ.

It was postulated that abrogation of the PFC response was caused by the quantitative neutralization of natural IFN-γ activity, which was required for PFC responses. Such a mechanism should be reversible upon the addition of excess IFN-γ. This was tested by adding purified recombinant-derived IFN-γ to

TABLE III

Dose Response Analysis of Inhibition of TRF Activity by Antibody to Mu IFN-γ and Recovery of Response by Addition of rIFN-γ

Source of rIFN-γ	Units of rIFN-γ added	No antibody	Direct PFC to SRBC per culture (SEM)		
			TRF(E-N) plus antibody to Mu IFN-γ		TRF(E-N) plus antibody to Hu IFN-γ
			25 U	125 U	125 U
Murine	0	0	457 (135)	247 (28)	1,085 (50)
	2.5	0	397 (58)	307 (93)	1,055 (107)
	25	17 (17)	450 (50)	360 (106)	1,787 (527)
	250	13 (12)	860 (111)	293 (47)	1,640 (280)
	2500	13 (9)	1,263 (142)	1,000 (27)	1,657 (303)
Human	0	0	497 (154)	327 (55)	1,277 (274)
	2.5	0	577 (88)	297 (29)	1,693 (124)
	25	6 (6)	953 (160)	247 (45)	1,467 (162)
	250	3 (3)	280 (26)	220 (30)	2,027 (155)
	2500	17 (17)	587 (55)	257 (29)	2,320 (183)
Controls: SRBC plus TRF(E-N)			1,474 (80)		
SRBC only			0		

T-depleted spleen cells were cultured for 4 d in the presence of SRBC and TRF(E-N). Antibody and/or rIFN-γ were added at the start of the cultures. Amounts of antibody and IFN-γ added are expressed in units per 0.2 ml culture.

TABLE IV

rIFN-γ Lacks TRF Activity and Does Not Complement Limiting Concentrations of TRF(E-N) for PFC Responses

Units of Mu rIFN-γ	PFC (SEM) per culture				
	Percent TRF(E-N) per culture				
	0	2.5	5	10	20
2,500	20 (12)	770 (106)	867 (96)	1,437 (108)	1,400 (202)
250	7 (7)	853 (87)	893 (107)	1,535 (81)	1,187 (78)
25	10 (6)	817 (64)	1,073 (47)	1,500 (126)	1,487 (94)
2.5	20 (10)	913 (62)	1,073 (108)	1,163 (251)	1,375 (54)
0	90 (25)	967 (52)	1,260 (51)	1,927 (155)	1,450 (130)

10^5 T-depleted spleen cells were placed in culture with 2×10^5 SRBC. TRF(E-N) and Mu rIFN-γ were added to the cultures as indicated. PFC were determined after 4 d. Results are expressed as mean PFC (SEM) per culture. Control values: None, 3 (2); plus TRF(E-N), 17 (5).

the cultures, as shown in Table II. The PFC responses which were supported by either murine or human TRF were strongly inhibited in a dose-dependent fashion by antiserum or purified antibody to IFN-γ. These inhibited responses were substantially or fully reconstituted by the addition of 2,500 U of *recombinant-derived murine IFN-γ.*

The stoichiometry of the anti-IFN-γ effect was examined in greater detail in a dilution analysis of both the antiserum to mo-IFN-γ and the r-IFN-γ which was added to the cultures. As shown in Table III, on comparing reversal of inhibitions caused by 25 U and 125 U of antiserum, recovery is more readily obtained with lower doses of antibody when a similar amount of IFN- is used. The addition of IFN-γ to TRF-containing cultures in separate experiments was found to have no effect on TRF-mediated responses. Suprisingly, IFN-γ also failed to augment weak PFC responses induced by suboptimal concentrations of TRF, suggesting that some other cytokine was limiting in the system, Tables IV and V.

It is also important to note in Table III that antiserum to human IFN-γ has little or no inhibitory activity, as also seen in Figure 2, and that human r-IFN-γ cannot reconstitute the inhibited response, although it significantly augments the PFC response of non-inhibited cultures. Thus the activity of human-derived TRF(E-N) cannot be explained by its content of human IFN-γ. The supernatant of the Gibbon lymphoma MLA-144 which constitutively produces IL-2, exhibited high TRF activity in these cultures as shown in Table V. Addition of antibody to mo-IFN-γ inhibited the PFC responses which were restored by addition of 2,500U of r-IFN-γ. No direct TRF effects of r-IFN-γ

TABLE V

Constitutive Production of TRF by a Primate Lymphoma MLA-144

Percent of MLA-144 Sn	PFC per culture (SEM)			
	Plus SRBC	Plus rIFN-γ and SRBC	Plus anti-IFN-γ and SRBC	Plus anti-IFN-γ, rIFN-γ, and SRBC
25	1,045 (45)	945 (205)	510 (70)	1,060 (128)
12.5	840 (108)	625 (60)	145 (47)	537 (223)
6.25	230 (60)	177 (102)	40 (10)	550 (125)
Controls				
None	2 (2)	—	—	—
TRF(E-N)	1,267 (60)	—	—	—

10^6 T-depleted spleen cells were cultured for 4 d in the presence of MLA-144 Sn. rIFN-γ (2,500 U) and/or RAM-IFN-γ (25 neutralizing units) was added to indicated cultures. Direct PFC were determined and the results expressed as the mean (SEM) of triplicate cultures.

TABLE VI

Inhibition of TRF Activity by Monoclonal Antibody to Mu IFN-γ and Recovery of the Response With rIFN-γ

Source of rIFN-γ (2,500 U)	Culture Sn dilution	Direct PFC (SEM) to SRBC per culture	
		Monoclonal R4-6A2 Culture Sn to Mu IFN-γ	Culture Sn of spent NS-1 cells
None	5×	87 (29)	1,163 (159)
None	1×	417 (78)	1,187 (73)
Murine	5×	1,140 (241)	ND*
Murine	1×	1,290 (145)	ND
Controls	SRBC plus TRF(E/N)	1,510 (105)	
	SRBC only	2 (2)	

T-depleted spleen cells were cultured for 4 d in the presence of SRBC and 20% TRF(E-N) *Sn from the monoclonal line R4-6A2 or from the myeloma P3/NS-1/1-Ag4-1* were used neat or were concentrated 5× using a Centricon 10 membrane (Amicon Corp., Danvers, MA). At the start of incubation some cultures also received Mu rIFN-γ

* Not done

TABLE VII

Human TRF(E-N) Supports Antigen-specific PFC Responses: Reversal by rIFN-γ of Antibody-induced Inhibition Is Also Antigen-specific

Antigen in culture	TRF in culture	Antibody and/or IFN-γ added	PFC per culture (SEM) measured on	
			SRBC	BRBC
None	TRF(E-N)	None	72 (26)	0
SRBC	TRF(E-N)	None	1,000 (79)	0
BRBC	TRF(E-N)	None	75 (18)	618 (48)
SRBC	TRF(E-N)	Anti-IFN-γ	500 (56)	8 (8)
BRBC	TRF(E-N)	Anti-IFN-γ	45 (15)	48 (10)
SRBC	TRF(E-N)	Anti-IFN-γ plus Mu rIFN-γ	955 (135)	0
BRBC	TRF(E-N)	Anti-IFN-γ plus Mu rIFN-γ	10 (6)	405 (65)
SRBC	TRF(E-N)	Mu rIFN-γ	878 (99)	2 (2)
BRBC	TRF(E-N)	Mu rIFN-γ	55 (11)	510 (58)
None	None	None	48 (10)	2 (2)
SRBC	None	None	15 (6)	0
BRBC	None	None	20 (7)	0

T-depleted spleen cells were cultured with 2×10^6 SRBC or BRBC for 4 d with additions to the cultures as indicated. 25 U of rabbit antibody or 2,500 U of rIFN-γ were used.

alone has ever been detected in these studies. Very recently a monoclonal antibody to mo-IFN-γ was generated (17) which can functionally neutralize the biological properties of IFN-γ. When added to the TRF assay this antibody showed potent inhibitory effects, as seen in Table VI, which were reversed by the addition of excess recombinant-derived murine IFN-γ.

Specificity of the PFC responses after reversal of antibody block by r-IFN-γ. The PFC responses supported by TRF(E-N) were shown in preliminary studies to be antigen-specific. However it was possible that the PFC responses produced by reversal of anti-IFN-γ antibody with excess r-IFN-γ were not antigen-specific, and could have been due to polyclonal B cell activation by r-IFN-γ. This was tested in an experiment using SRBC or BRBC antigens as seen in Table VII. The results show that both the uninhibited and the recovered responses are fully antigen specific.

TRF induces IFN-γ production and potent NK activity in T-depleted spleen cell cultures. The preceeding experiments suggest that IFN-γ is produced in cultures of T-depleted murine spleen cells. This was directly tested using supernatants from spleen cells cultured in various conditions as shown in Table VIII. The data demonstrate that IFN-γ is produced only in cultures stimulated with TRF(E-N) and can be detected on day 3, although levels which are lower than the sensitivity of the antiviral assay may be present at earlier times. Antiserum to IFN-γ, but not to IFN-γ, , abolished both the antiviral activity of the IFN and the PFC response.

Since B cell populations are not thought to produce IFN-γ we examined the extent of non-B cell contamination in the spleen cell cultures. At the start of culture fewer than 1% (generally 0-0.6%) Thy-1.2$^+$ cells are routinely detected but by day 4, 5-10% of the cells are Thy-1.2$^+$. Interestingly, these cells are also sIg$^+$ suggesting an atypical T cell lineage (Table A). Since natural killer (NK) cells and their precursors 1. show variable expression of Thy-1 (24) and, 2. have been shown to proliferate and mature in cultures of T-depleted spleen cells in the presence of IL-2 (24) and, 3. have been shown to produce abundant IFN-γ on stimulation with human (25) or murine (26) IL-2, we examined the PFC cultures after 4d for NK cell activity. As shown in Table IX a dramatic appearance of NK cells is evident. This effect requires stimulation of the cultures with TRF. Lysis of YAC-1, but not P-388D$_1$ target cells confirms the NK character of the effector cells. Thus, TRF(E-N) induces both IFN-γ production and the striking appearance of NK cell activity of T-depleted murine spleen cells.

Time of action of IFN-γ in the TRF assay. To investigate the time of action of human TRF(E-N) and of IFN-γ in this system, PFC responses were measured after adding TRF(E-N) at different times and also upon varying the time of addition of antibody or the time of reversal of inhibition with r-mo-IFN-γ. As shown in Table X, TRF(E-N) is most active when included in the cultures from the beginning of culture or from d1. Similarly antibody to mo-IFN-γ was active to inhibit responses only if added very early to the cultures. When antibody to mo-IFN-γ was added on d1 only a partial inhibition was obtained. Finally, in

TABLE VIII

TRF(E-N) Induces IFN-γ in T-depleted Spleen Cell Cultures

Culture conditions	Units* of IFN-γ				Mean (SEM) PFC per culture
	Day of assay				
	1	2	3	4	
Medium	0	0	0	0	0
SRBC	0	0	0	0	0
TRF(E-N)	0	0	0	160	0
TRF(E-N) + RAM-IFN-γ	0	0	0	0	5 (5)
TRF(E-N) + SRBC	0	0	160	160	717 (88)
TRF(E-N) + SRBC + RAM-IFN-γ	0	0	0	0	17 (5)

Control TRF(E-N) in the absence of cells, 0 U IFN

10^6 T-depleted spleen cells were cultured for variable periods of time in the presence of TRF, antigen, and antibody to IFN-γ, as indicated. Supernatants were pooled from triplicate wells and assayed for IFN-γ levels. On day 4, cells from individual wells were assayed for PFC responses. RAM-IFN-γ was added to indicated cultures at 25 neutralizing units per culture. IFN activity was measured by inhibition of vesicular stomatitis virus cytopathic effects on L$_{929}$ cell monolayers using serial twofold dilutions of supernatant. IFN-γ was identified in each of the active supernatants by sensitivity to pH 2.0, and neutralization with monoclonal R4-6A2 antibody and RAM IFN-γ serum, but not with RAM-IFN-γ antibody or antibody to Mu IFN-α, -β. The assays were standardized by inclusion of a control Mu IFN-α, -β preparation (NIH reference reagent No. G002-904-511).

* 1 U of IFN as determined in this assay is equivalent to 0.08 IU/ml.

TABLE IX

TRF(E-N) Induces the Maturation of Potent NK Activity

Exp. No.	Target cell	Lytic units before culture	Lytic units after culture with:			
			None	SRBC	TRF(E-N)	SRBC plus TRF(E-N)
1	YAC-1	2	0.5	0.6	667	1,000
2	YAC-1	4	7	5	196	147
	P-388D$_1$	ND	0	4	11	4

NK cell activity was examined in assay cultures using 10^6 YAC-1 or P-388D$_1$ target cells labeled with ^{51}Cr. Results are expressed in lytic units per 10^7 effector cells, as described in Materials and Methods.

TABLE X

Kinetic Analysis of TRF Action and Inhibition With Antiserum to Mu IFN-γ

Addition to cultures		PFC (SEM) to SRBC per culture with variable component added on days			
Continuously present	Variable component	0	1	2	3
Nil	Nil	0			
Nil	rIFN-γ	6 (5)	0	0	3 (3)
Nil	TRF(E-N)	2,157 (18)	2,153 (63)	429 (107)	9 (3)
TRF(E-N)	Nil	1,917 (237)			
TRF(E-N)	RAM-IFN-γ	275 (48)	1,153 (13)	2,017 (108)	2,077 (47)
TRF(E-N) plus RAM-IFN-γ	Nil	427 (52)			
TRF(E-N) plus RAM-IFN-γ	2,500 U rIFN-γ	2,017 (110)	1,913 (185)	1,170 (119)	577 (26)

T-depleted (DBA/2 × CBA/N)$_1$ spleen cells were cultured with SRBC for 4 d. At the start of the culture or at various times thereafter the indicated additions were made.

cultures where responses were inhibited by antibody to IFN-γ, recovery of responses was obtained only when r-mo-IFN-γ was added during the first two days of culture. This last result shows that the specific IFN-γ component of signalling for TRF effects must be received early in the response and is the likely mechanism underlying the kinetics obtained in the first two groups of results.

The inhibition of TRF-supported responses by antibody to IFN-γ, is apparently caused by a delay in the kinetics of the PFC response as seen in Table XI. This result is expected if endogenous IFN-γ production by the spleen cell population can saturate the capacity of the inhibitory antibody and if a critical period for IFN-γ action has not lapsed.

TABLE XI

Antibody to IFN-γ Delays the Generation of PFC

Source of TRF	Anti-body to IFN-γ	PFC per culture (SEM)			
		Day of assay			
		3	4	5	6
TRF(F-N)	–	10 (6)	767 (171)	1,530 (91)	1,125 (105)
TRF(F-N)	+	0	137 (72)	1,160 (57)	1,240 (77)
Con A Sn	–	0	977 (221)	1,090 (58)	55 (25)
Con A Sn	+	0	153 (62)	750 (91)	990 (228)
No TRF	–	0	0	7 (3)	7 (7)

10^6 T-depleted spleen cells were cultured in the presence of the indicated source of TRF and 2×10^6 SRBC. 25 neutralizing units of RAM-IFN-γ were added to some cultures. Specific PFC were determined on the day indicated. Results are expressed as the mean PFC (SEM) per culture.

DISCUSSION

In this report we have examined the role of IFN-γ in TRF-dependent PFC responses of murine spleen cells to red blood cell antigens. Several antibody preparations with high specific neutralizing activity for human and murine IFN-γ were added to the cultures to test their effects on TRF action. The results show that antibody to murine but not to human IFN-γ can abrogate the TRF activity of either human, primate or murine TRF preparations and that these inhibitions are reversed specifically by the addition of excess murine but not human recombinant-derived IFN-γ.

Although TRF activity was described more than a decade ago (27) there had been little progress in the molecular identification of the active factor(s) until recently, probably because several distinct factors cooperate to mediate TRF effects, as shown by new evidence. In addition to SRBC responses, TRF is active in antibody responses of B cells to hapten-protein antigens (12) to thymus-independent antigens (13,28) and in polyclonal activation of B cells by anti-Ig antibodies (4). Thus TRF appears to be the major cytokine-controlled regulatory pathway for the expansion and maturation to antibody-producing cells of a major subpopulation of B cells which are Lyb-5$^+$ (29). Using new methods for lymphokine purification as well as supernatants of long-term or cloned T cell lines in factor-reconstitution experiments, clear evidence for the presence of several required components in TRF has emerged (3,8-10). Collectively, the data indicate that for the systems studied, at least two major components are involved; first, an IL-2 containing supernatant of T cells, which however may include additional mediators, and second, an IL-2-depleted supernatant of Con A-activated spleen cells (8), which may also be replaced by secreted products of some T cell lines (10,11,30). In some studies, IL-2-containing supernatants alone are active as TRF, which may be due to the activity of this lymphokine on contaminating T cells or other non-B cells (2); in other systems however TRF activity of IL-2 alone is not generally reported. Recent work has shown that the second component has an apparent molecular weight of 40KD (8) and that its activity is most evident when added 24h after the start of cell cultures (11,12), as noted previously for unfractionated supernatants of Con A-stimulated spleen cells (31). This second component was shown to be sensitive to treatment at pH 2 and to correlate with IFN-γ activity in supernatants of T cell lines (1,12,14). In a recent report r-IFN-γ showed similar biological activity (15). IFN-γ is already known to act as a differentiation signal in several murine immunological systems such as the induction of MHC antigens (32), the activation of mononuclear phagocytes (17,18,33) the enhancement of natural killer cell activity (25) and a TRF-like action in the genesis of cytotoxic T cells (34), however the regulation of B cell differentiation by IFN-γ represents a new and important biological function of this lymphokine.

To examine the role of IFN-γ in antibody responses supported by TRF, we used the same preparations of antibody to IFN-γ which have been shown to functionally neutralize the anti-viral and MAF activity of IFN- (17,18). Other antibodies to IFN-γ have been shown to abrogate TRF help for cytotoxic T cells (35) and recently the induction of MHC class II antigens by IFN-γ was

also shown to be inhibited by antibody to IFN-γ (19). In a general sense, the power of an approach using antibody-mediated inhibition is that it can demonstrate a necessary role for a specific lymphokine in a mixture of biologically active molecules present in a crude supernatant. In the present study this method could determine whether or not IFN-γ is a necessary component of TRF action and whether this is true for a variety of TRF preparations. While previous reconstitution studies, which used monoclonal cytokines or recombinant-derived proteins, support a role for IFN-γ it is not clear that IFN-γ is normally a required component of TRF. It is alternatively possible that IFN-γ may substitute for, or bypass the need for other mediators present in TRF which normally support antibody responses. The results in the present study consistently show that strong, specific inhibitions are obtained using four independent sources of TRF, demonstrating that IFN-γ is a lymphokine which is consistently required for the activity of TRF.

A human IL-2-rich lymphokine fraction, known to have TRF activity on human B cells (36) was shown in the present study to be an effective TRF for murine B cells and to behave similar to murine TRF in terms of magnitude, kinetics and specificity, in the support of murine PFC responses to SRBC. Moreover, this preparation was inactive with B cells from mice with the xid immunological defect as shown for murine TRF (6), suggesting that common TRF pathways are followed. In view of the need for IFN-γ in the action of murine TRF as well as the species specificity known for the biological actions of IFN-γ (18), it was surprising that human-derived TRF was active in this system. However, as the data show, human IFN-γ appears to have no role in these responses, since rabbit antibody to human IFN-γ and four monoclonal antibodies with high hu-IFN-γ neutralizing activity (not shown) were inactive to supress TRF activity while antibody to mo-IFN-γ inhibited PFC production. The specific lymphokine target of this antibody was examined in several ways. First, it was found that antiserum to mo-IFN-γ had no effect on human IL-2 and BCGF in their respective functional assays (not shown). Second, it was shown that the inhibited responses were restored by the addition of excess r-IFN-γ of murine but not human origin. Third, an antigen affinity-purified rabbit immunoglobulin, specific for r-mo-IFN-γ, was similarly inhibitory. This indicates that the inhibitions could be fully explained by antibodies to r-mo-IFN-γ rather than other antibodies in the sera. Fourth, a monoclonal antibody which specifically neutralizes the antiviral effect of mo-IFN-γ also abrogated TRF activity and this block was relieved with excess mo-IFN-γ. This result argues most strongly that mo-IFN-γ is the target lymphokine of the antibodies since it is less likely that a monoclonal antibody would avidly cross-react with another lymphokine, which is a possibility to be considered with the polyvalent antisera. The finding that r-hu-IFN-γ did not restore the response agrees not only with the specificity of the antisera but also with the species specificity of its biological effects, e.g. it is devoid of any antiviral activity when tested in murine assays (18). Moreover, in other experiments, the functional activity of TRF(E-N) was unaltered following exposure to pH 2.0.

The reversal of inhibition of PFC responses upon addition of r-IFN-γ suggests that quantitative relationships in this system may be complex. It appears that greater than 250 U of IFN-γ are required to reverse the inhibition

produced by 25 neutralizing U of antibody. This descrepancy may occur because IFN-γ which is added at the initiation of the cultures may be substantially degraded or absorbed in the high density cell cultures prior to its time of action, which may occur from 24-72 h. Time-dependent absorption of IFN-γ in murine cell cultures has been demonstrated (37).

Together the above results argue that IFN-γ is necessary in TRF action, even in the case of a TRF which genetically lacks this factor and, that in the latter circumstance, it is produced by the cultured T-depleted murine spleen cells. Recent work on the production of IFN-γ by spleen cells has shown that unfractionated spleen cells (38) as well as nu/nu or conventional T-depleted spleen cells (25) produce IFN-γ upon culture in the presence of purified murine or human IL-2. NK cells appear to be a very adequate source of this factor (26). In the present study, significant levels of mo-IFN-γ were recovered from the culture supernatants after 3-4 days. Since some biological systems, e.g. MAF assays are considerably more sensitive to IFN-γ than are antiviral assays, (18) it is possible that IFN-γ concentrations adequate to support B cell responses are present at earlier times. This possibility is favoured by the high local concentrations of factor which would occur in cell to cell interactions. It also appears that the IFN-γ is not produced constitutively but is induced by TRF. The cellular source of this factor remains unclear from our studies but may be the T-related large granular lymphocytes which possess NK activity and are reduced but usually not fully eliminated in most T cell depletions. These cells may also comprise the population component which permits TRF activity of FS6-14.13 supernatants (2) or may constitute the novel helper cells postulated by Parker to explain a possible mode of action of IL-2 in TRF-supported PFC responses in the anti-Ig system (10). Residual T cell activity in T-depleted spleen cell populations has been well documented (10,39). In the present study, high levels of NK cells were produced after 4d of culture in the presence of TRF(E-N). In view of the ability of these cells to produce IFN- (25,26) they may play a crucial role in TRF- mediated B cell responses. In related experiments we found that antibody to IFN-γ did not permanently inhibit TRF-supported anti-SRBC responses but rather delayed the appearance of PFC (Table XI). The similar magnitude of the intact and delayed responses suggests that the antibody acts as a transient sink for IFN- produced in the cultures, and that the antibody capacity is soon surpassed by endogenous IFN-γ production.

In other experiments an IL-2-rich supernatant constitutively produced by the Gibbon T cell line MLA-144, supplied by Dr. H. Rabin, when used at a similar dilution of IL-2 activity was found to be similar to TRF(E-N) for TRF activity. This result argues that any potentially co-purified but undetectable mitogens are likely not responsible for the biological effects of TRF(E-N). Interestingly, r-mo-IFN-γ showed no TRF activity in these experiments either alone (Tables III and X) or in the presence of suboptimal concentrations of TRF(E-N) or TRF(MLA-144) (Tables IV and V). This suggests that either IFN-γ is not limiting in the latter cultures or alternatively that r-IFN-γ is functionally inactive but antigenically intact. The latter possibility seems unlikely since the molecule is functional in several independent biological assays. The lack of TRF activity of IFN-γ alone is consistent with the need

for at least two factors for TRF activity, the second of which may be supplied by the IL-2-rich TRF(E-N).

Our data do not address the role of IL-2 itself in these TRF-dependent responses. IL-2 may act in synergy with IFN-γ, or it may secondarily induce the production of IFN-γ (25,26) and perhaps other factors such as BCGF (30,40). Alternatively it is possible that other factors such as BCGF which co-purify with human IL-2 (41) are directly involved in these responses. Human IL-2 is known to be active on murine T cells and has been shown to replace murine IL-2 in both cell expansion and antibody production by T-depleted murine spleen cells (10). While most studies used IL-2-containing supernatants of T cell lines, which may contain other factors such as BCGF (1,10,42,43) which may be needed for maximal responses, the affinity-purified human IL-2 used by Parker, argues most strongly for a role of IL-2 in the TRF system. This role may be direct and necessary, such as a signal for B cell differentiation or may be indirect, such as the induction of other factors as considered above which are the ultimate mediators of signals needed for B cells. The production of secondary mediators induced by IL-2, or by TRF as shown in the present study, may account for some of the apparent discrepancies concerning the controversial role for IL-2 in TRF action. As discussed in recent reports (3,28), such effects could be variable and may depend significantly on the purity of the B cells and of the factors used. A recently described intermediate modulator for Ia antigen expression induced by IFN-γ is an example of this concept (19). Some of these uncertainties could be resolved in studies with monoclonal antibodies which neutralize the activity of specific lymphokines such as BCGF, IL-1 and IL-2. Alternatively, antibodies to the cellular receptors for these lymphokines may serve the same purpose and further help to identify the cell subsets involved. Single cell assays may be the most useful approach to analyze the distinct action of lymphokines on B cells. Such cultures would be free of the products of all forms of accessory cells.

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